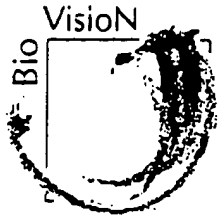

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Hannover, 23-Okt-01

Sehr geehrter Herr Dr. Meyers,

anbei eine Declaration bezüglich der DPD-Patentanmeldung USA.

Ich habe zwei Beispiele für DPD mit Bildmaterial angeführt, deren technische Grundlage bereits 1996 gegeben war (Chromatographie, UV-Messung, MALDI-MS).

Weiterhin habe ich versucht, die vom Examiner aufgeworfenen Fragen durch Definitionen und Beispiele zu beantworten.

Ich hoffe, dass Sie hiermit für die amerikanischen Anwaltskollegen hinreichende Unterstützung haben, um im Sinne einer positiven Fortführung des Verfahrens zu handeln.

Bitte zögern Sie nicht, sich bei Rückfragen direkt an mich zu wenden.

Mit freundlichen Grüßen

Dr. Peter Schulz-Knappe

BioVisioN GmbH & Co. KG

Declaration for DPD-Patent

The one skilled in the Art is enabled to follow the scope of the patent after studying the enablement given in the Patent.

This enablement is explained in two different examples by using the following technologies:

1. HPLC-Separation of complex peptide mixture from biological sources (A: Mouse -plasma, B: Urine
2. Measurement of peptides by UV -trace or mass spectrometry (MALDI) of collected fractions
3. Relation of results from the samples to a reference (Differential Peptide Display)

Questions of the examiner/Definition of terms

Peptides:
(low molecular weight) peptides are all peptides present within the sample. Their origin may differ with respect to the kind of sample that is analysed. If human plasma is analysed, peptides are of human origin such as fragments of plasma proteins, peptide hormones, etc. If the patient suffers from a severe bacterial infection, plasma could contain peptides from bacterial origin. If liver cells from a patient with viral Hepatitis are analysed, the sample could contain, among liver cell peptides, also peptides from virus.

Reference:
The reference is the counterpart of the sample which shall be analysed. The differential comparison is based on the comparison of every single peptide from the sample with the corresponding peptide from the reference. Please see the examples from the knock-out mouse (+/+) and (-/-). Under certain circumstances, it may become necessary to use several individual samples and compare these differentially to several individual references. In these cases, the selection of the different individual samples and/or references has to be representative. For instance, a number of 10 caucasian males with colon cancer (prior to Treatment) may form the group the blood sample is taken from. Then, these 10 caucasian males after surgery and removal of tumor are forming the group the blood reference is taken from. The reference is the defined control.

Dipeptides:
The invention aims at the analysis of peptides. There is no international standard definition of the term peptide concerning the length of the amino acid ch ain. In our view, any combination of amino acids linked by a peptide bond up to a molecular mass range of 10 to 30 kDa is a peptide. Thus, the smallest peptide consists of the combination of two amino acids via a peptide bond. This is a dipeptide. Since pe ptides are composed out of more than 20 different amino acids, there is a high number of possible combinations to form dipeptides. The example gly-gly refers to the dipeptide with the lowest molecular mass.

Fractions:

A fraction in terms of chromatograp hy is a part of the effluent recovered during a separation step. Usually, several fractions are collected (e.g. collection of the effluent every minute during a 1 hour separation will result in 60 fractions). Fractions usually contain different subsets of peptides from the sample. Depending on the complexity of the peptide content of a sample, the sample may be used as is or may be fractionated by using various biochemical methods such as chromatography. Then the fractions are analysed to measure peptides.

Organism:
An organism in the scope of the invention is any living thing, may it be multicellular such as a plant, a mouse, a human being, or single cell organism such as a bacterium or a fungus. In all cases, comparison of peptides from a sample from said organisms (urine or blood, blood cells or tissue specimen or a leaf, or the entire organism such as the bacterium itself) to the peptides from the reference gives those peptides that are indicative for the differentiation between sample and reference. For commercial use, these peptides are useful as identifiers of the respective condition and they can be tracked for in additional specimen of the same kind for which it is not known whether they belong to the sample or to the reference group (or to neither of them).

Condition:
The actual status (condition) of an organism at the time of sample production (such as drawing of blood) is reflected in the sample that conserves this status similar to a snap shot picture. This snap shot picture represents the condition. If the sample is from a sick patient, this condition may be considered „bad condition", if it is from a pregnant woman, it may be considered „good condition".

Measurement of peptides „free of hypothesis":
In the four examples the selection of sample and reference is not meant to be free of hypothesis. The sample selection carries the hypothesis, that peptides that are in principle not known to the researcher are indicative to describe the condition.
The analysis of the results from the measurement s is not meant to be „free of hypothesis". All (qualitative and/or quantitative) differences in peptide composition are considered to be relevant.
The measurement of peptides shall be „free of hypothesis".
This will be explained in the following.
In contrast to the work by Jimenez and Harry, applicants invention does not request preformulated ideas that direct the researcher to only those peptides that are already known to be relevant in the condition of the organisms of interest. Jimenez explicitly exclu des the analysis of unknown compounds and the drawing of conclusions from these compounds in his work. For his analysis, only those peptides that are explained by his hypothesis (to be involved in salt metabolism and to be parts derived from a certain precursor peptide). Harry directs his measurements to a specific protein, the HIV p24 antigen, and neglects all other possible peptides or proteins in the sample on purpose. By doing so, they follow the routine application of peptide analysis.
Current knowledge shows, that metabolic compounds of an organism can be used to analyse the „condition" of said organism. This is done routinely by e.g. drawing blood from patients and measuring blood glucose, cholesterol, liver and cardiac enzymes in case of certain conclusions the examiner draws from his patient´s condition. In general, a hypothesis gives the reason for any measurement, e.g. there is the suspicion of the examiner that the patient may be a diabetic,

therefore glucose is selected for measurement. Or the patient had chest pain and the examiner is suspicious that the underlying disorder is a myocardial infarction, therefore cardiac enzymes are measured. Or the patient is suspected to suffer from an HIV -infection, therefore HIV p24 antigen is measured. In all these instances, there is a clear cut and scientifically proven hypothesis, exactly which compounds shall be measured. All compounds measured are known to the examiner. No compounds are measured, that are not known, and if they were measured, their measurement is not used in interpretation of the „condition". This is equivalent to Jimenez´work.
The invention does not use a hypothesis regarding to what known peptides shall be measured in order to analyse the condition of the respective organism. Applicants´ invention aims at the definition of such condition by analysis of peptides from samples drawn from both sample and reference and the link of selected of the measured peptides (one, two, or many; in principle unknown by gene or amino acid sequence and function) to either the one condition or the other. Those peptides found to differ among those conditions are considered to be relevant, those peptides that are identical are considered to be not relevant for the description of the condition of those organisms.
This is novel and innovative.

A set of examples is given to illustrate the terms organism, sample and reference. Reference is used as control and may be composed out of a group of individual analyses.

Pregnant female (Organism: human, Condition: pregnant)
versus
non-pregnant female
(Sample: Plasma)

Transgenic animal (Organism: Mouse, Condition: manipulated gene)
versus
non-Transgenic animal
(Sample: Plasma or Tissue)

45 year old caucasian male with colon cancer prior to Treatment (Organism: Human, C ondition: oncologic disorder)
versus
same person after surgery and removal of tumor
Sample: Plasma, Urine, Tears...)

bacteria in culture treated with a drug (Organism: Escherichia coli, Condition: Drug stimulated)
versus
bacteria in culture
(Culture supernatant or cell extract)

All examples show two organisms of the same kind with conditions that are different. The condition of said organisms is not necessarily indicative for a „disease" (as pregnancy or the response to a drug is not a disease) but is valued as being characteristic for the individual sample composition. Characteristic differences are present in these examples on e.g. the presence or absence of a tumor, the presence or absence of a gene (and ist action on the organism), the

presence or absence of pregnancy and the presence or absence of drug treatment. Applicants' invention aims at the definition of such condition by analysis of peptides from samples drawn from both members of these pairs and the link of selected (one, two, or many; in principle unknown by gene or amino acid sequence and function) of the measured peptides to either the one condition or the other. Those peptides found to differ among those conditions are considered to be relevant, those peptides that are identical are considered to be not relevant for the description of the condition of those organisms.

Application scenario 1

A Peptide Display of blood -plasma from a knock-out mouse (sample -/ -) is produced.
Steps:

1. Peptide extraction from Plasma by means of precipitation o f proteins, centrifugation of pellet and recovery of peptides from the supernatant
2. Separation of peptide mixture by means of conventional high performance liquid chromatography and collection of 96 fractions during chromatography
3. MALDI-Mass measurement of all fractions to analyse all peptides in each fraction
4. Combination of mass spectra to a peptide mass map to illustrate peptide composition of sample
5. Relation of results to a reference (mouse without knock-out; +/+) by comparing individual peptide signals and extraction of differentially expressed peptides (steps 1-4 are performed also for the reference mouse)

The sample is the knock-out (-/ -), the reference is the mouse without knock-out (+/+). The condition of the organism (mouse) is: There is one gene missing, therefore this mouse has a specific condition that differs from its healthy brothers and sisters. Whether this is a bad or a good condition is not relevant. Whether this is a pathologic condition or not is not relevant. What is relevant is that there is a clear difference between sample and reference that is relevant for the condition.

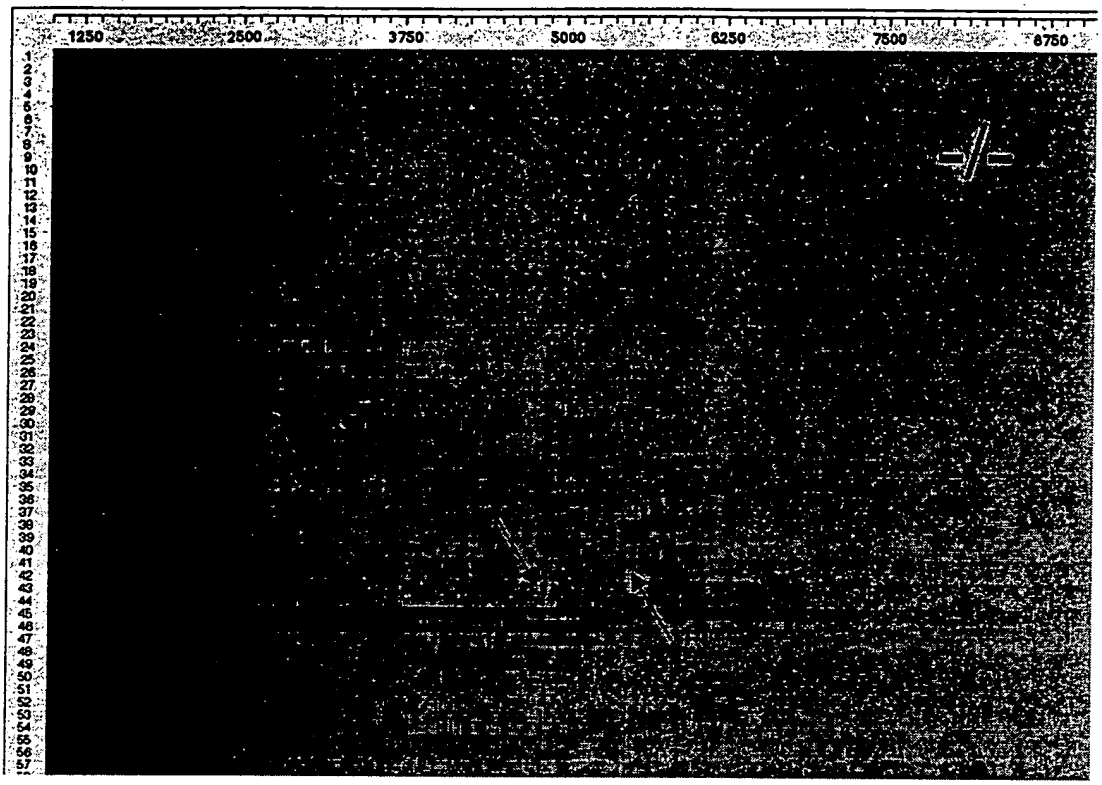

Fig. 1a: Peptide Mass Map from sample. Measurement of Peptides from knock-out mouse (-/-) plasma. Technology base: Mouse plasma peptides were separated by chromatography into fractions. All peptides in the fractions were analysed by Mass spectrometry. Brown bars represent more than 1.000 individual peptides. Green arrows point at peptides which are present in the knock-out mouse (-/-) and reduced or absent in the control animal (+/+). Black arrows point at peptides which are absent in the knock-out mouse (-/-) and present in the control animal (+/+).

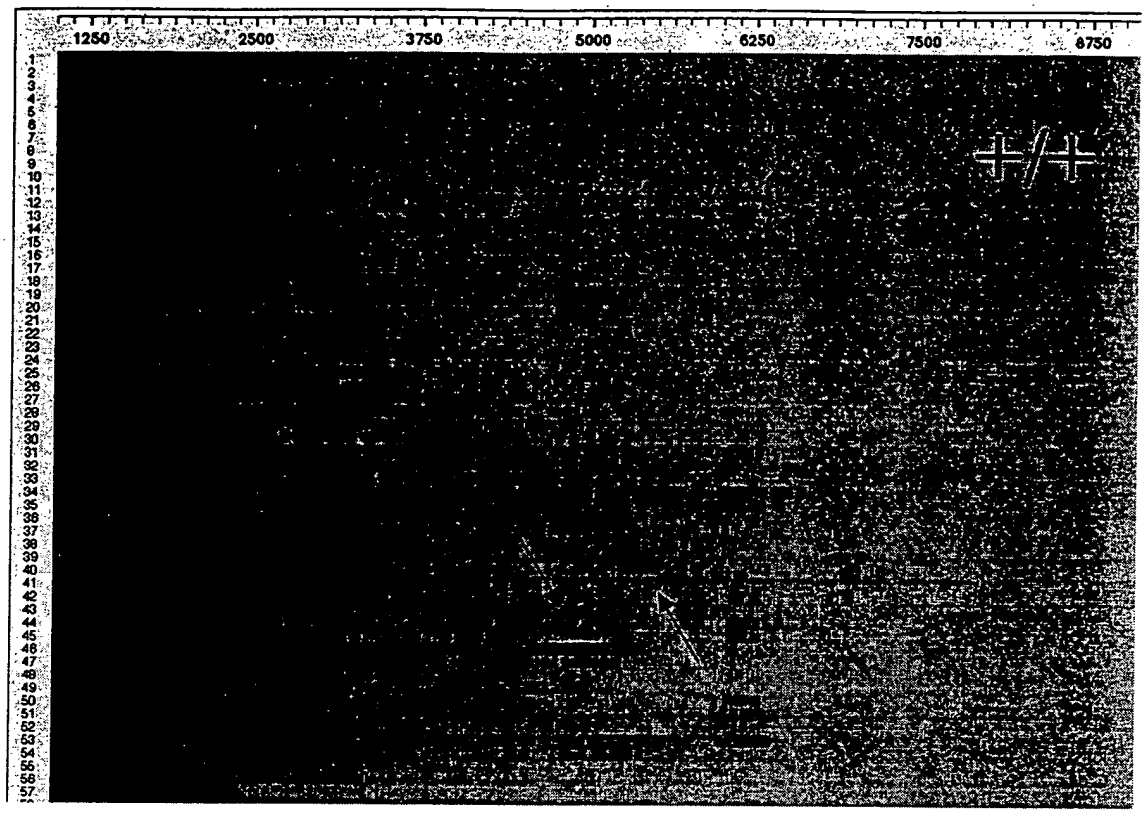

Fig. 1b: Peptide Mass Map from reference. Measurement of Peptides from control mouse (+/+) plasma. Technology base: Mouse plasma peptides were separated by chromatography into fractions. All peptides present in the fractions were analysed by Mass spectrometry. Brown bars represent more than 1.000 individual peptides. Green arrows point at peptides which are present in the knock-out mouse (-/-) and reduced or absent in the control animal (+/+). Black arrows point at peptides which are absent in the knock -out mouse (-/-) and present in the control animal (+/+).

Fig. 1 c: Single MALDI Mass Spectra from the same fractio n obtained after chromatography of plasma peptide extract from reference (control, upper trace) and sample (knock-out, lower trace). In this case, two peptides are present in the sample that are absent in the control. However, the measurements show other p eptides that are present in both traces.

Fig. 1 a, b and c show a collection of Mass spectrometry measurements that are combined to represent a 2-dimensional diagram (1 a and b). Hundreds to thousands of peptides are displayed. Most or all of these peptides are not known by sequence or by function. There was no hypothesis during the measurement to detect a specific peptide of known sequence or function (as did Harry et al. by specifically analysing samples for the presence f HIV p24-Antigen or Jimenez et al. for detecting specific peptides from a precursor known to be involved in salt -physiology). What we can state is that the removal of one gene has led to significant changes in the peptide pattern (exemplified by those peptides with an arrow; 1 a, b, c).
This example shows: By measuring peptides without a hypothesis (to specifically measure one or some known peptides) and relating those measurements to a reference the scope of the invention is shown. Peptides not previously known to be present in either s ample or reference are now of value to distinguish between sample and reference.

The commercial use of this information is as follows: We are able to take one or more of these differential expressed peptides, obtain their sequence (by using methods known to the one skilled in the art), and use them, e.g. as diagnostics use to detect the knock-out condition in other individuals.

Application scenario 2

A Peptide Display of human blood-plasma (sample) is produced.

Steps:

1. Peptide extraction from Plasma by means of precipitation of proteins, centrifugation of pellet and recovery of peptides from the supernatant
2. Separation of peptide mixture by means of conventional high performance liquid chromatography (HPLC) and collection of 96 fractions during chromatography
3. Hypothesis-free MALDI-Mass measurement of all fractions
4. Combination of mass spectra to a peptide mass map to illustrate peptide composition of sample
5. Relation of results to a reference (non-treated) by comparing all individual peptide signals (UV-Trace from HPLC and/or MALDI -mass measurements) and extraction of differentially expressed peptides

The Applicants Invention is used to give an important advantage by assessing peptides in a sample with technologies capable of doing so (such as UV-detection, mass spectrometry, NMR, others). Ideally, all peptides would be analysed in a qualitative (such as: precise molecular mass) and quantitative (concentration) way. Nevertheless, also the analysis of a certain number of peptides may serve the scope of the invention. This is the case, if the analysis is capable to depict peptide signals in a differential way, i.e. show the difference between two (ore more) samples.

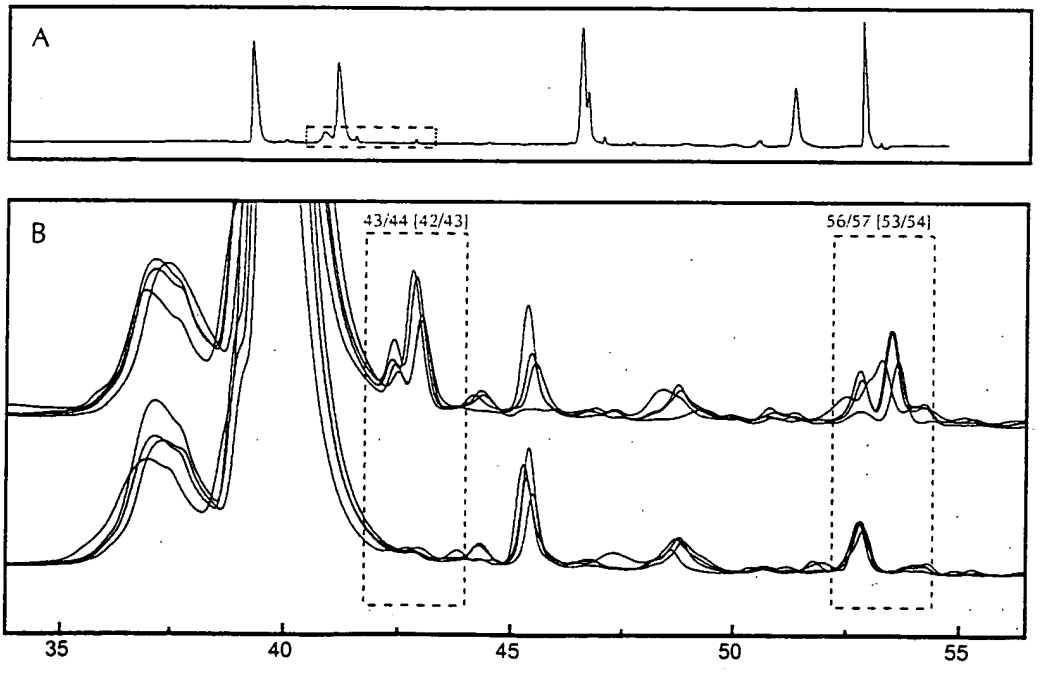

Fig. 2a: UV-trace of HPLC-experiments from peptide extracts from human blood plasma samples. A: Full view of one chromatography with insert (dashed line). B: zoom view of insert area from A with differential comparison of 4 samples (black line, patients were treated) and 4 references (red line, patients were not treated). Despite the high overall similarity, three distinct differences are depicted in the blue areas where, during minute 43 and 54, the sample contains peptides

different than in the reference. The peptides are not known but indicate the „condition" of the organism.

The next figure shows the Mass spectrometry of a fraction collected during the chromatography.

A

B

Fig. 2b: MALDI-MS measurements from the second blue area depicted in Fig. 2a. The peptide with molecular mass 3952.4 is present in much higher co ncentration in A and therefore detected as the difference between sample and reference. The peptide is not known but indicates the „condition" of the organism.